The Role of Intrinsic Pathway Apoptosis via Caspase-9 in Atherogenesis Due To Atherogenic Diet in Sprague Dawley Rats

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ABSTRACT

Thirty male rats, strain Sprague Dawley were used as experimental animal to study the role of intrinsic (mitochondrial) pathway apoptosis in atherogenesis due to high fat and high cholesterol diet. The rats were randomly alloted into three group (I, II, III) of 15 each. Group I as control was fed normal diet, group II was fed diet containing high fat diet, and group III was fed containing high fat and high cholesterol diet (atherogenic diet). After 6 and 12 weeks on experimental diet, 15 rats were selected randomly (5 rats of each group). All animal were then killed and the aorta were taken out for caspase-9 immunohistochemical analysis. Based on the present study result it can be concluded that high fat diet and high cholesterol diet could induced apoptosis through caspase-9.

INTRODUCTION

Apoptosis, or programmed cell death, is a physiological process of cellular autodestruction. Apoptosis plays critical roles in development, maintenance of homeostasis, and host defense in multicellular organism (Ucker, 1991; Walker *et al.*, 1988; Wyllie *et al.*, 1991). Dysregulation of this process is implicated in various disease, such cancer (Williams, 1991) Alzheimer's disease, and various degenerative disease including atherosclerosis (Duke *et al.*, 1996; Thompson, 1995).

Apoptosis occurs in two pathways (intrinsic apoptotic pathway and extrinsic apoptotic pathway) and can be initiated by a variety of different factors, both from internal or or external factors (Jacobson, 1997). Intrinsic apoptotic pathway or mitochondrial pathway initiated by activation of the pro-apoptotic members of the Bcl-2 family leads to altered mitochondrial membrane permeability resulting in release of cytochrome c into the cytoplasma. In the cytoplasma, or on the surface of mitochondria, cytochrome c is boud by the protein Apaf-1 (apoptotic protease activating factor), which also binds caspase-9, and ATP. Cytoplasma cytochrome c then forms, together with Apaf-1 and caspase-9, an apoptosome to orchestrate activation of other caspases and the biochemical execution of apoptosis (van der Heiden et al., 1997; Geng, 2001).

Caspase-9 or ICE-like apoptotic protease 6 (ICE-LAP 6) or mammalian CED-3 homologue 6 (Mch6) is a member of subfamily CED-3, and similar to caspase-3, although differently in the active site of pentapeptida. According to Cardone et al., (1997), caspase-9 activity is regulated by phosphorylation. Active form of caspase-9 could activated caspase effector like caspase-3, caspase-6, and caspase-7 (Zou *et al.*, 1997; Srinivasula *et al.*, 1998).

Several studies with both animals and humans, involving *in vivo* and *in vitro* assay or administration of dietary lipids and cholesterol, have recently described an important role of several fatty acid in the induction of apoptosis (dePablo *et al.*, 2002). Different mechanisms of action have been proposed in order to explain the action of fatty acids on apoptosis modulation. Polyunsaturated fatty acids have been defined as substances capable of inducing cell death via a mitochondrial process. According to Yano *et al.*, (2000), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), taken together with vitamin E could reduce apoptosis in vitro due to TNF-Ü stimulation. Another studies demonstrated that affect of PUFA in apoptosis is through mitochondria (dePablo *et al.*, 1999), or by downregulation of Bcl-2 (Avula et al., 1999). Another fatty acid, like palmitic acid, induced apoptosis in cell culture directly in vitro mediated by mitochondria (dePablo *et al.*, 1999).

Certain oxysterols have been shown to be cytotoxic in vitro and the mode of toxicity has been identified as apoptosis in certain cell lines (Rusinol *et al.*, 2004). Christ *et al.* (1993) showed that 25-OH and 7â,25-dihydroxycholesterol (7,25-OH) induced cell death in murine lymphoma cells in vitro and in mouse thymocytes. Cell death induced by these oxysterols exhibited many characteristics of apoptosis such as DNA fragmentation, considered to be the hallmark of apoptotic cell death.

This present study is expected to add information the role of fat and cholesterol in diet against apoptotic pathway (mitochondrial pathway/ intrinsic pathway) in atherogenesis on rats animal models Sprague Dawley.

MATERIALS AND METHODS

Thirty male Sprague Dawley rats, 100-150 grams of body weight were used as experimental animals. They were housed individually, and then randomly assigned to three diets group with five rats in each group. Tap water was freely available. Group I as control was fed diet containing normal fat and cholesterol, group II was fed diet containing high fat and normal cholesterol, and group III was fed diet containing high fat and high cholesterol. After 6 and 12 weeks on experimental diet, 15 rats were selected randomly (5 rats of each group) and then were sacrified and aorta were taken out for imunohistochemical analysis.

Analytical methods

Immunohistochemistry was carried out on 5 µm section of formalin-fixed parrafin-embedded tissue using streptavidin-biotin technique. The technique was divided into 4 step, (1) the tissue sections were deparaffinized and rehydrated (2) washed with H_2O_2 to remove endogen peroxidase, and the incubated in microwave for 10 minutes. After washed with phosphate buffer saline (PBS) for 10 minutes, tissue sections were incubated with blocking serum (Santa Cruz, Biotechnology, USA) solution for 10 minutes. Removes excess serum from tissue section, and applied primary antibody (antibody anti caspase-9) (BioVision Research Products) without washing, followed by incubation at room temperature for 45 minutes, then washed with phosphate buffer saline (PBS) for 10 minutes, (3) tissue section were incubated with biotinylated secondary antibody (Santa Cruz, Biotechnology, USA) at room temperature for 10 minutes, washed with PBS for 10 minutes, and incubated with streptavidin-peroxidase conjugate for 5 minutes. After washed with PBS for 10 minutes, tissue sections were incubated with 3,3' diaminobenzidin (Santa Cruz, Biotechnology, USA) solution for 15 minutes, washed with aquadest for 10 minutes, and (4) applied counterstain hematoxyline-eosin (Zymed Laboratory Inc, Carlton Court, San Francisco, USA) for 3 minutes, washed with aquadest and applied mountuing medium Mayer's egg albumin (Zymed Laboratory Inc, Carlton Court, San Francisco, USA) for microscopic examination (Microscope digital camera system, Olympus DP 12) (Wasito, 1997; Trieb *et al.*, 2003)

RESULTS AND DISCUSSION

The result after 6 and 12 weeks on experimental diets on immunohistochemical analysis of caspase-9 are presented on table 1.

Table 1. The result after 6 and 12 weeks on experimental diets on immunohistochemical analysis of caspase-9 among group I, group II, and group III.

Group	Time periods			
	No	6 weeks	No	12 weeks
Group I	1	Negative (-)	16	Negative (-)
Group I	2	Negative (-)	17	Negative (-)
Group I	3	Negative (-)	18	Negative (-)
Group I	4	Negative (-)	19	Negative (-)
Group I	5	Negative (-)	20	Negative (-)
Group II	6	Negative (-)	21	Negative (-)
Group II	7	Positive (+)	22	Negative (-)
Group II	8	Positive (+)	23	Negative (-)
Group II	9	Negative (-)	24	Positive (+)
Group II	10	Negative (-)	25	Positive (+)
Group III	11	Negative (-)	26	Negative (-)
Group III	12	Negative (-)	27	Positive (+)
Group III	13	Negative (-)	28	Positive (+)
Group III	14	Positive (+)	29	Positive (+)
Group III	15	Positive (+)	30	Negative (-)

Immunohistochemical analysis of aorta showed positive caspase-9 on the rats fed high fat diet (group II) and fed high fat and high cholesterol (group III). caspase-9 were found on atheromatous plaque rats number 7 and 8 (high fat diet for 6 weeks) (figure 1) rats number 24 and 25 (high fat diet for 12 weeks) (figure 2), rats number 14, 15 (high fat and high cholesterol diet for 6 weeks), and rats number 27, 28, 29 (high fat and high cholesterol for 12 weeks) (figure 3). However, caspase-9 were not found on rat number 6, 9, 10 (high fat diet for 6 weeks), rats number 21, 22, 23 (high fat diet for 6 weeks), rats number 11, 12, 13 (high fat and high cholesterol for 6 weeks), and rats number 26 and 30 (high fat and high cholesterol for 12 weeks) (figure 4).

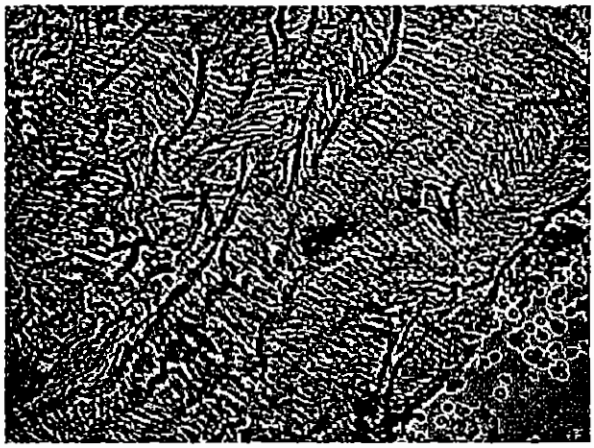

Fig.1. Aorta from Sprague dawley rats fed high fat diet 6 weeks on experimental diet. Colored brown of Caspase 9 was observed (A) in atheromatous plaques (SB staining, 500 X.).

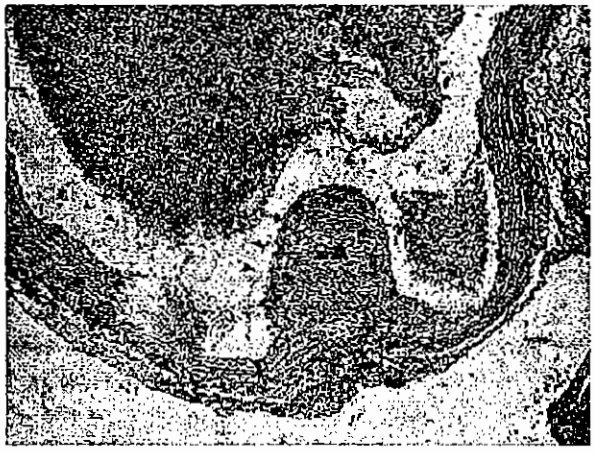

Fig.2. Aorta from Sprague dawley rats fed high fat diet 12 weeks on experimental diet. Colored brown of Caspase 9 was observed (A) in atheromatous plaques (SB staining, 250 X.).

Fig.3. Aorta from Sprague dawley rats fed high fat diet 12 weeks on experimental diet. Colored brown of Caspase 9 was observed (A) in atheromatous plaques (SB staining, 500 X.).

Fig.4. Aorta from Sprague dawley rats fed high fat diet 12 weeks on experimental diet. Colored brown of Caspase 9 was not found in atheromatous plaques (SB staining, 100 X.).

The finding caspase-9 indicate that apoptotic pathway in this research through intrinsic pathway (mitochondrial pathway) and may caused by high fat and high cholesterol diet. The increased of cholesterol concentration may caused apoptosis caspase-9 as an initiator caspase. Of the biological attributable to oxysterols, the one that has received most attention, over the last couple of decades, is their ability to induce apoptosis in a variety of cell lines *in vitro* (Ryan *et al.*, 2005).

The result supported the previous studies that oxysterol could induced apoptosis through intrinsic pathway. The ability of oxysterols to induce apoptosis through the intrinsic pathway has been well studied (Ryan *et al.*, 2005). 7-ketocholesterol has been shown to induce apoptosis via release of cytochrome c from the mitochondria with subsequent caspase-9 activation in a variety of cell lines (Lizard *et al.*, 1998). Miguet-Alfonsi *et al.* (2002) have found that 7â-hydroxycholesterol (7â-OH) and ketocholesterol could induced apoptosis *in vitro* through loss of mitochondrial potential membrane. The lost of mitochondrial potential membrane followed by the released of cytochrome c (Leonarduzzi *et al.*, 2004; Seye *et al.*, 2004). According to Slee *et al.* (1999), the release of cytochrome c from the intermembrane space is the commitment step in both the intrinsic and extrinsic pathways. In the cytosol, cytochrome c interacts with apoptotoic protease-activating factor-1 (apaf-1) to form apoptosome and then recruits and activates procaspase-9. Activated caspase-9 in turn cleaves the effector caspase-3, -6, and -7 to execute apoptosis (Zeiss, 2003). Moreover, Lim *et al.* (2003) examined 7â-OH and 25-OH-induced cell death in THP cell line. They determined that apoptosis induced by the two oxysterols proceeded *via* activation of caspase-9.

Acoording to Panini and Sinensky (2001), intrinsic pathway (mitochondrial pathway) could induced vascular cells apoptosis. In this case, the release of caspase-9 is probably mediated by the death receptor Fas. Fas is an important death receptor in the vascular system. According to Gibbons and Pollman (2000), the FasL-induced cell-execution pathway seems to be reinforced by the capacity to promote parallel activation of the proapoptotic factor Bid by proteolytic cleavage. This activation of Bid stimulates cytochrome c release from the mitochondria, apaf-1 activation, and caspase-9 cleavage, and thereby results in caspase-3 stimulation via caspase-9-dependent pathway.

In this study, caspase-9 were not detected in rats number 6, 9, 10 (high fat diet for 6 weeks), rats number 21, 22, 23 (high fat diet for 6 weeks),

rats number 11, 12, 13 (high fat and high cholesterol for 6 weeks), and rats number 26 and 30 (high fat and high cholesterol for 12 weeks). The negative result of caspase-9 may caused by Fas signal from death receptor that failed to induce Bid, which is then were not activeted mitochondria. Another possibility is probably due to two distinct type of death receptor CD95. Type I is characteristic by high level of DISC formation that would increased caspase-8 levels. In contrast, type II is characteristic by low level of DISC formation that would decreased caspase-8 activity (Scaffidi *et al.*, 1998). In this study, type CD95 signal may be blocked by Bcl-2 family members like Bcl-2 and Bcl-xL as an antiapoptotic factor, thus failed to form apoptosome complexes (Willis *et al.*, 2003). The result supported by Yang *et al.* (1997) suggested that overexpression of Bcl-2 will blocked the release of cytochrome c from mitochondria thus inhibited apoptosis.

CONCLUSION

Based upon the experimental result, it can be concluded that high fat diet and high cholesterol diet could induced apoptosis through intrinsic (mitochondrial pathway) apoptosis via caspase-9.

ACKNOWLEDGEMENT

The author would like to thank to Mr Dhirgo Adji for their co-assistance, Mr Yuly, Mr Daliyo for their technical assistance. This research was funded by BPPS, Directorate General of Higher Education, Department of Education and Culture.

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